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Major cold shock protein of *Escherichia coli*

(gene regulation/stress response/heat shock response)

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ABSTRACT **When exponentially growing *Escherichia coli* cell cultures were transferred from 37°C to 10°C or 15°C, the production of a 7.4-kDa cytoplasmic protein (CS7.4) was prominently induced. The rate of CS7.4 production reached 13% of total protein synthesis within 1–1.5 hr after a shift to 10°C and subsequently dropped to a lower basal level. Regulation of CS7.4 expression was very strict, such that synthesis of the protein was undetectable at 37°C. We have cloned the gene encoding this protein and have completed the nucleotide sequence analysis, which revealed that the gene encodes a hydrophilic protein of 70 amino acid residues.**

The heat shock response in *Escherichia coli* has been well characterized (for review, see ref. 1). When cell cultures are transferred from 30°C to 42°C, 17 heat shock proteins are transiently expressed. This induction has been shown to be accomplished primarily by an alternate σ subunit of RNA polymerase (σ^{32}), encoded by *rpoH (htpR)* (2, 3), which recognizes specific heat shock promoters (4). Other stresses besides heat, including ethanol treatment (5), DNA-damaging agents such as nalidixic acid and UV irradiation (6), and bacteriophage infection (7), have been shown to induce all or part of the heat shock response.

It has been shown that *E. coli* also responds to cold temperatures (8). When growing cell cultures were transferred from 37°C to 10°C, 13 polypeptides, none of which are heat shock proteins, were shown to be induced. Seven of these polypeptides were identified as NusA, RecA, dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase, polynucleotide phosphorylase, pyruvate dehydrogenase (lipoamide), and initiation factors 2α and 2β. Twelve of the polypeptides exhibited a 2- to 10-fold induction of synthesis after the shift to cold temperature; however, one polypeptide, designated F10.6, was reported to undergo the greatest induction at cold temperature.

In this study we were able to identify a 7.4-kDa protein (CS7.4) which, although undetectable at 37°C, is rapidly induced upon shift to 10°C or 15°C. The gene for CS7.4, designated *cspA*, was cloned and its DNA sequence was determined.‡ A preliminary result indicates that protein CS7.4 may play a role in protecting cells from damage due to freezing. A possible mechanism for tight regulation of the gene for protein CS7.4 by temperature will be discussed.

MATERIALS AND METHODS

Bacterial Strains and Media. *E. coli* strain SB221 (*lpp hsdR trpE5 lacY recA*/F′ *lacI^q lac$^+$ pro$^+$*; ref. 9) was used as the host cell in the CS7.4 induction experiments and for chromosomal DNA used for Southern analysis. Strain JM83 [*ara Δ(lac-proAB) rpsL φ80 lacZΔM15*; ref. 10] was used for harboring the pUC9-derived plasmids. Strain JM103 [*Δ(lac,pro) supE thi strA endA sbcB15 hsdR4* F′ *traD36 proAB lacI^q ZΔM15*; ref. 11] was used for preparing single-stranded DNA for sequencing.

Cultures that were pulse-labeled were grown in M9 minimal medium supplemented as described (12). Cultures used for two-dimensional gel electrophoresis and chromosomal DNA preparation were grown in L broth (13).

Induction Experiments. All cultures were grown to a density of 2×10^8 cells per ml in a 10-ml culture prior to temperature shift. For pulse-labeling, usually a 1.1-ml sample was removed from the culture to a beaker containing 10 μCi of [^{35}S]methionine (Amersham, >1000 Ci/mmol; 1 Ci = 37 GBq) or Trans^{35}S-label (ICN). For the CS7.4 protein stability experiment described in Fig. 3, 6.2 ml of culture was transferred to a beaker containing 50 μCi of Trans^{35}S-label (ICN) and chased with nonradioactive methionine (final concentration, 80 μg/ml) and cysteine (final concentration, 100 μg/ml). All samples were collected by centrifugation and the pellets were dried by lyophilization. SDS/polyacrylamide gel electrophoresis was carried out as described (14). The concentration of polyacrylamide in the resolving gel was 17.5%.

The intensity of bands on the autoradiograms was determined using a Hoefer model GS 300 scanning densitometer. The rate of CS7.4 synthesis relative to total protein synthesis was determined by integrating the peak for the CS7.4 band and dividing by the sum of all the integrated peaks in a particular lane.

Two-Dimensional Electrophoresis and Protein Sequencing. A 10-ml culture was grown to a density of 2×10^8 cells per ml at 37°C and transferred to 14°C for 4 hr. Cells were then harvested and fractionated for the soluble fraction as described (15). A trace of protein, pulse-labeled for 30 min after a shift to 15°C as described above, was then mixed with 250 μg of soluble-fraction protein. Two-dimensional electrophoresis was then performed with isoelectric focusing in the first dimension [ampholines, pH 3–10, 1.5% (wt/vol); pH 6–8, 0.5%) and SDS/polyacrylamide gradient gel electrophoresis (10–18.4% acrylamide/2.7% *N,N′*-methylenebisacrylamide) in the second dimension as described in the method of O'Farrell (16). Separated protein was electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore) using a semidry blotter apparatus (Sartorius) and 48 mM Tris/39 mM glycine/1.3 mM SDS/20% (vol/vol) methanol, pH 9.2, as the transfer buffer. The membrane was then stained for protein with Coomassie blue R-250, dryed, and subjected to autoradiography. The autoradiogram clearly identified a heavily labeled protein at approximately the same molecular weight observed for CS7.4. The autoradiographic spot was used to identify the CS7.4 spot on the stained membrane, which was then excised from the blot. Automated Edman degradation was performed directly on the stained

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30139).

membrane fragment by using the method of Matsudaira (17) and an Applied Biosystems model 470 gas-phase sequencer.

Cloning and Sequencing. pUC9 plasmid digested with *Hin*dIII was ligated with chromosomal DNA digested with *Hin*dIII from fraction 7 of the Southern blot (see Fig. 4) using T4 DNA ligase. The 2.4-kilobase (kb) *Hin*dIII fragment containing the gene for CS7.4, which had been cloned into pUC9, was isolated from pUC9 by digesting with *Hin*dIII and separated on a 5% polyacrylamide gel. The fragment was then subcloned into M13 and DNA sequencing was done using the chain-termination method (18). DNA sequencing was accomplished using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate and the enzyme Sequenase as described by the manufacturer (United States Biochemical).

Chromosomal DNA was prepared as described (19). Southern blot analysis was carried out as described by Southern (20). Recombinant DNA techniques including DNA fractionation was done as described by Maniatis *et al.* (21).

RESULTS

Induction and Stability of the Major Cold Shock Protein. Aliquots of an exponentially growing cell culture at 30°C were transferred to 42°C, 25°C, or 18°C and were immediately pulse-labeled with [^{35}S]methionine for 10 min. Samples were then subjected to polyacrylamide gel electrophoresis and the resulting autoradiogram is shown in Fig. 1. A protein with an apparent molecular mass of 8 kDa was observed in the protein pattern produced after a shift to 25°C or 18°C. No corresponding band was seen in the preshift or 42°C-shifted cultures. Cell fractionation indicated the cold-induced protein was found almost exclusively in the cytoplasmic fraction (data not shown). We have designated this protein, CS7.4.

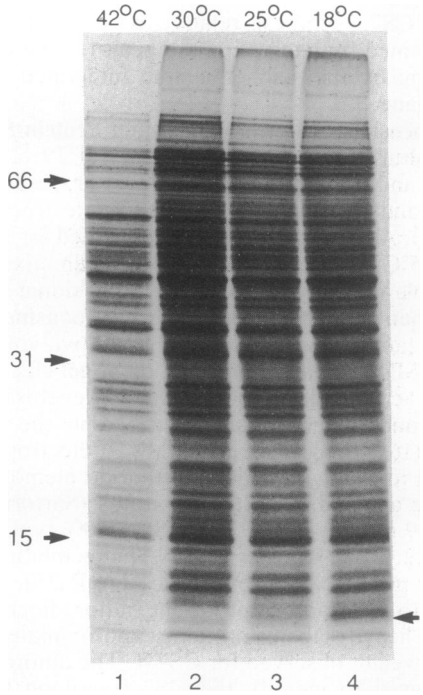

FIG. 1. Major cold shock protein induction. Aliquots of a cell culture growing at 30°C were transferred to 42°C, 30°C, 25°C, and 18°C as indicated and immediately pulse-labeled with [^{35}S]methionine for 10 min. Samples were subjected to SDS/polyacrylamide gel electrophoresis and the autoradiogram is shown here. Molecular mass standard sizes are indicated on the left in kDa. The arrow on the right indicates the induced major cold shock protein.

The kinetics of CS7.4 induction was studied by a pulse-labeling experiment. Cells were labeled with [^{35}S]methionine for 30-min periods after increasing periods of exposure to cold temperature. The autoradiogram in Fig. 2*A* shows that within the first 30 min after shift to either 10°C or 15°C, there was a sudden induction of CS7.4 production. As shown in Fig. 2*B*, induction was transitory with both the time of maximal induction and the maximal rate of synthesis dependent on the temperature. After a shift to 15°C, the maximal rate of synthesis was approximately 13.1% of total protein synthesis and was reached 30–60 min after the shift (Fig. 2*A*, lane 10). Shift to 10°C gave a maximal rate of synthesis of approximately 8.5% of total protein synthesis 60–90 min after the shift (Fig. 2*A*, lane 4). Subsequently, in both cases, the rate of synthesis of CS7.4 dropped off, ultimately reaching one-fifth of the maximum for the 15°C-shifted culture. This pattern of induction is reminiscent of the heat shock response.

The stability of protein CS7.4 was studied in the following manner. A cell culture growing at 37°C was transferred to 15°C and then pulse-labeled with radioactive methionine during the second half-hour after temperature shift. The culture was then chased with nonradioactive methionine for various lengths of time. The results indicate that 20 hr after synthesis at 15°C only approximately 30% of CS7.4 had been degraded (data not shown).

Protein CS7.4 was also found to be fairly stable at 37°C. A cell culture was chased for an hour with nonradioactive methionine at 15°C after a pulse and then transferred to 37°C for 1.5 hr. There was no discernable decrease in the amount of radioactive protein (data not shown).

Cloning of the Gene Encoding CS7.4. To synthesize a DNA probe complementary to the gene encoding CS7.4, a partial amino-terminal sequence of the protein was obtained. A cell culture was shifted to 14°C for 4 hr and the soluble fraction was subjected to two-dimensional electrophoresis (16) and then bands were electrophoretically transferred to a polyvinylidene difluoride membrane. The Coomassie blue-stained membrane is shown in Fig. 3. A spot corresponding to the molecular mass observed for CS7.4 is present on the gel. This spot did not appear in a soluble fraction prepared from a similar culture grown at 37°C with no temperature shift and comigrated during two-dimensional electrophoresis with a spot heavily labeled with [^{35}S]methionine at 14°C (data not shown). The stained spot was excised from the membrane and automated gas-phase Edman degradation was performed directly on the stained membrane fragment. A polypeptide was detected with the following amino acid sequence: Ser-Gly-Lys-Met-Thr-Gly-Ile/Leu-Val-Lys-Trp-Phe-Asn-Ala-Asp-Lys-Gly-Phe-Gly-Phe-Ile-Xaa-Pro, where Xaa is unknown and Ile/Leu indicates that both isoleucine and leucine were identified (64% and 36%, respectively) in the seventh cycle. A mixed-degenerate oligonucleotide probe was then made to match a short region of the amino acid sequence as shown below:

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                Lys-Trp-Phe-Asn-Ala
Probe: 5′-AAR-TGG-TTY-AAY-GC-3′,
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where R is a purine and Y is a pyrimidine.

For Southern blot analysis, *E. coli* chromosomal DNA was digested with various restriction enzymes, including *Sal* I, *Bam*HI, *Pst* I, *Eco*RI, and *Hin*dIII. The autoradiogram from Southern blot hybridization with the mixed oligonucleotide probe is shown in Fig. 4. Each digest gave at least one distinct band. In particular, the *Hin*dIII digest (lane H) yielded one band of 2.4 kb. A *Hin*dIII digest of chromosomal DNA was then fractionated by gel electrophoresis and each fraction was subjected to Southern blot analysis with the probe. As can be seen in Fig. 4, DNA fragments from fractions 7 and 8 clearly hybridized with the probe, which corresponds well

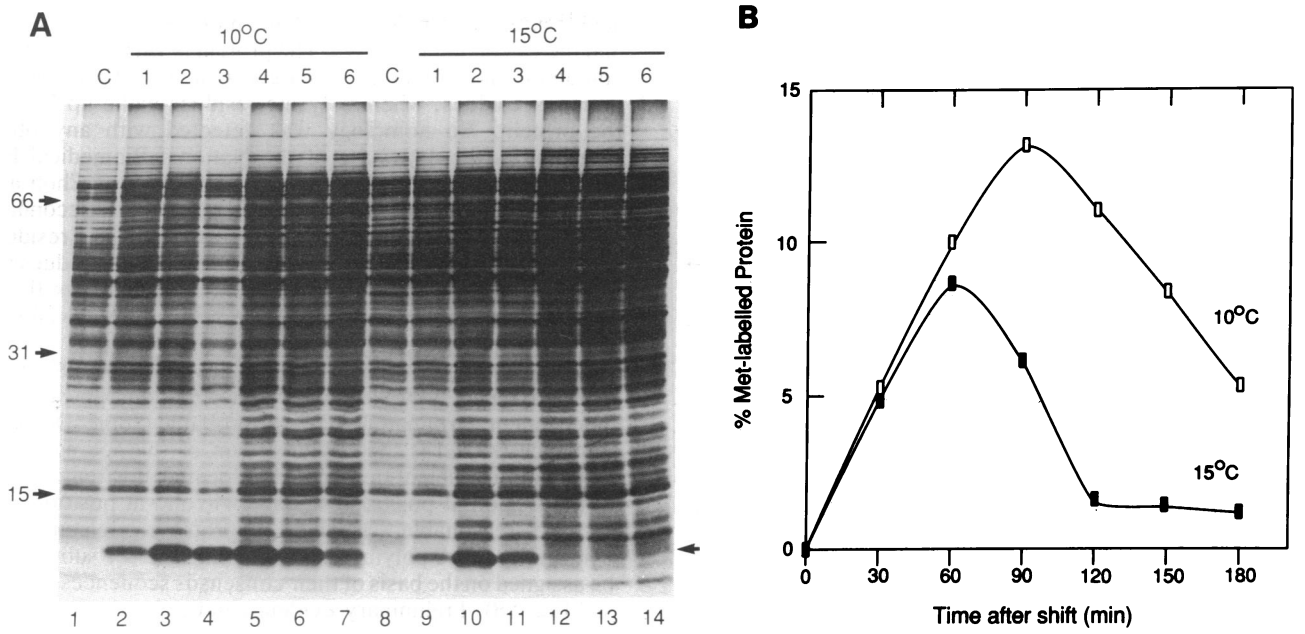


FIG. 2. Transient induction of CS7.4. Cell cultures growing at 37°C were transferred to either 10°C or 15°C as indicated. Aliquots were then pulse-labeled with [^{35}S]methionine for 30-min periods and electrophoresed as described in Fig. 1. (*A*) C indicates a control where an aliquot was pulse-labeled for 5 min at 37°C before the temperature shift. Pulse-labeling periods after temperature shift are as follows. Lanes: 1, 0–30 min; 2, 30–60 min; 3, 60–90 min; 4, 90–120 min; 5, 120–150 min; 6, 150–180 min. Arrows are as in Fig. 1. (*B*) The autoradiogram in *A* was subjected to scanning densitometry and the percent methionine-labeled CS7.4 protein in the whole cell was determined. Each time point on the graph is the quantitation for the percent methonine-labeled CS7.4 protein at the end of each 30-min labeling period. For example, between 30 and 60 min (60-min time point on the ordinate), CS7.4 accounted for 10% of the total methionine-labeled protein in the cell at 10°C. The 0-time point accounts for the 5-min pulse at 37°C, which is described in *A*.

with the 2.4-kb *Hin*dIII band in the original chromosomal digest. DNA from fraction 7 was subsequently ligated into the *Hin*dIII site of pUC9. *E. coli* transformants harboring a chromosomal fragment complementary to the probe were identified by filter hybridization. One of the positive clones was designated pJJG01.

DNA Sequence of the Gene Encoding CS7.4. The strategy of the nucleotide sequence analysis as well as a restriction map

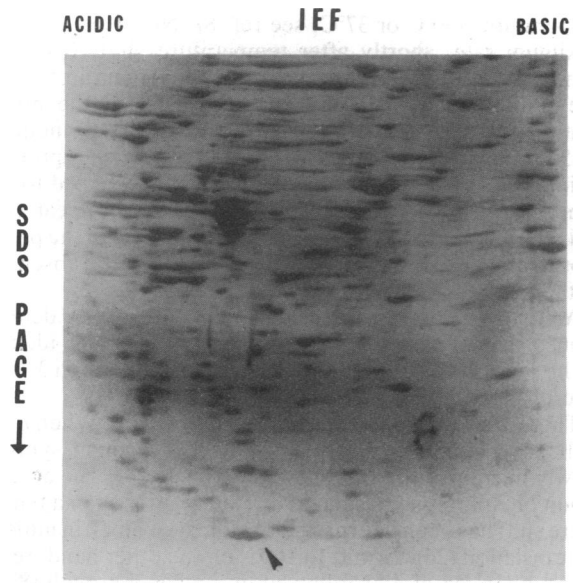


FIG. 3. Two-dimensional gel utilized in purification of CS7.4. A cell culture growing at 37°C was transferred to 14°C for 4 hr. The culture was then harvested and fractionated, and the cytoplasmic fraction was subjected to two-dimensional gel electrophoresis. The first dimension is isoelectric focusing and the second dimension is SDS/polyacrylamide gel electrophoresis. The gel was electroblotted onto a polyvinylidene difluoride membrane and stained with Coomassie blue dye. Arrowhead indicates CS7.4

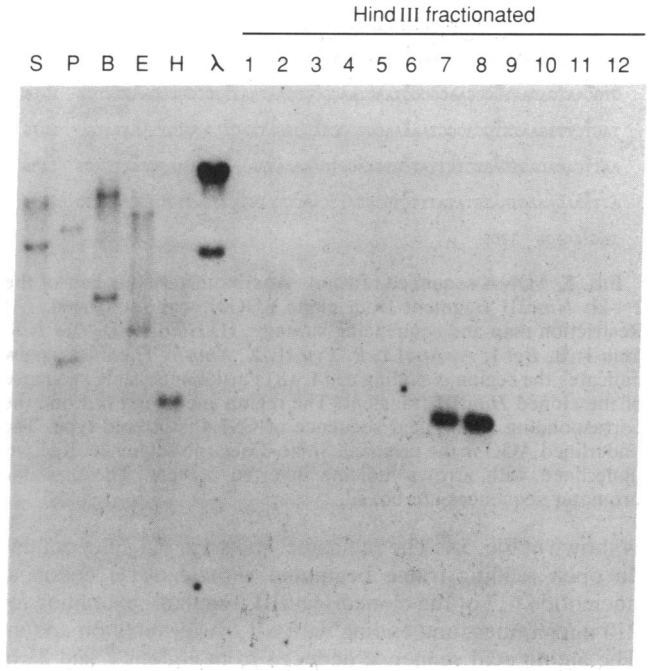
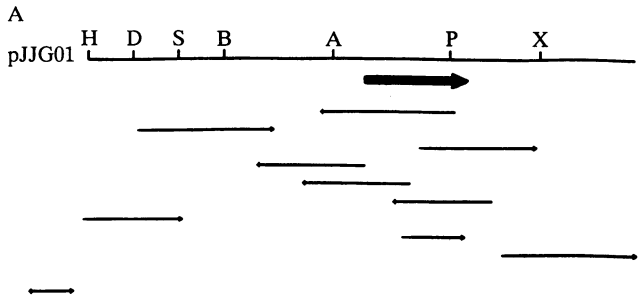


FIG. 4. Southern blot analysis for *cspA*. *E. coli* chromosomal DNA was digested with various restriction enzymes and the DNA was transferred from an agarose gel to a nitrocellulose sheet. Hybridization was carried out using the degenerate probe described in the text, and the autoradiogram is shown. Restriction enzymes used for digests are as follows. Lanes: S, *Sal* I; P, *Pst* I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; λ, λ DNA digested with *Hin*dIII, which was used as a size standard. Chromosomal DNA digested with *Hin*dIII was also fractionated by agarose gel electrophoresis, and fractions 1–12 are shown (lanes 1–12).



B

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AAGCTTCGATGCAATTCACGATCCCGCAGTGTGATTTGAGGAGTTTTCAATGGAATATAA   60

AGATCCAATGCATGAGCTGTTGAGCAGCCTGGAACAGATTGTTTTTAAAGATGAAACGCA  120

GAAAATTACCCTGACGCACAGAACAACGTCCTGTACCGAAATTGAGCAGTTACGAAAAGG  180

GACAGGATTAAAAATCGATGATTTCGCCCGGGGTTTTGGGCGTATCAGTCGCCATGGTAAA  240

GGAATGGGAATCCAGACGCGTGAAGCCTTCAAGTGCCGAACTAAAATTGATGCGTTTGAT  300

TCAAGCCAACCCGGCATTAAGTAAGCAGTTGATGGAATAGACTTTATCCACTTATGCTGT  360

TTACGGTCCTGATGACAGACCGTTTTCCAACCGATTAATCATAAATATGAAAAATAATTG  420

TTGCATCACCCGCCAATGCGTGGCTTAATGCACATCAACGGTTTGACGTACAGACCATTA  480

AAGCAGTGTAGTAAGGCAAGTCCCTTCAAGAGTTATCGTTGATACCCCTCGTAGTGCACA  540

TTCCTTTAACGCTTCAAAATCTGTAAAGCACGCCATATCGCCGAAAGGCACACTTAATTA  600

TTAAAGGTAATACACT ATGTCCGGTAAAATGACTGGTATCGTAAAATGGTTCAACGCT  658
                 MetSerGlyLysMetThrGlyIleValLysTrpPheAsnAla

GACAAAGGCTTCGGCTTCATCACTCCTGACGATGGCTCTAAAGATGTGTTCGTACACTTC  718
AspLysGlyPheGlyPheIleThrProAspAspGlySerLysAspValPheValHisPhe

TCTGCTATCCAGAACGATGGTTACAAATCTCTGGACGAAGGTCAGAAAGTGTCCTTCACG  778
SerAlaIleGlnAsnAspGlyTyrLysSerLeuAspGluGlyGlnLysValSerPheThr

ATCGAAAGCGGCGGCTAAAGGCCCGGCAGCTGGTAACGTAACCAGCCTGTAA TCTCTGC  836
IleGluSerGlyAlaLysGlyProAlaAlaGlyAsnValThrSerLeu -

TTAAAAGCACAGAATCTAAGATCCCTGCCATTTGGCGGGGATTTTTTTATTTGTTTTCAG  896

GAAATAAATAATCGATCGCGTAATAAAATCTATTATTATTTTTGTGAAGAATAAATTTGG  956

GTGCAATGAGAATGCGCAACGCCGTAAGTAAGGCGGGAATAATTTCCCGCCGAAGACTCT 1016

TACTCTTTCAATTTGCAGGCTAAAAACGCCGCCAGCTCATAACTCTCCTGTTTAATATGC 1076

AATTCACACAGTGAATCTCTTATCATCCAGGTGAAAAATAAAAGCGTGAAACAAATCACT 1136

ATTAAAGAAAGTAATCTATATTTCTGCGCATTCCAGCTCTGTGTTGATTTCACGAGTATG 1196

TACTGCACC 1205
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FIG. 5. DNA sequence of *cspA*. Approximately one-half of the 2.4-kb *Hin*dIII fragment from clone pJJG01 was sequenced. (*A*) Restriction map and sequencing strategy. H, *Hin*dIII; D, *Dra* I; S, *Sma* I; B, *Bgl* I; A, *Apa*LI; P, *Pvu* II; X, *Xmn* I. The thick arrow indicates the region encoding *cspA*. (*B*) Partial nucleotide sequence of the cloned *Hin*dIII fragment. The region encoding *cspA* and the corresponding amino acid sequence of CS7.4 is in bold type. The underlined AGG is the probable Shine–Delgarno sequence. Regions underlined with arrows indicate inverted repeats. The probable promoter sequences are boxed.

is shown in Fig. 5*A*. The sequence, shown in Fig. 5*B*, contains an open reading frame beginning with an ATG codon at nucleotide 617 of the cloned *Hin*dIII fragment, extending for 210 nucleotides, and ending with a TAA termination codon. The amino acid sequence deduced from codons 2 and 23 of this open reading frame agrees completely with the sequence determined by Edman degradation. Furthermore, the size of the predicted polypeptide is consistent with the observed size of CS7.4. We, therefore, conclude that this open reading frame is the coding region of the gene responsible for CS7.4 synthesis. We designate this gene *cspA*. The data also indicate that the initial methionine residue was cleaved off after synthesis.

The sequence reveals that the gene codes for a 70-amino acid residue protein. The calculated molecular weight is 7402 and the calculated pI is 5.92. The protein is very hydrophilic, containing more than 20% charged residues. There are no arginine residues, whereas lysine residues make up 10% of the protein. No homology was detected with any other sequence in the data base of the National Biomedical Research Foundation (release no. 59). The rules of Chou and Fasman (22) did not reveal any predominant secondary structural conformation; however, if the amino acid residues are plotted on a helical net, 12 of the 16 charged residues are adjacent as oppositely charged pairs. This suggests that a large portion of the protein may be in an α-helical conformation.

Downstream of the gene, from nucleotides 857 to 878 is an inverted repeat followed by a T-rich region typical of ρ-independent terminators (23). A probable Shine–Delgarno sequence (AGG) is located upstream of the coding region beginning at nucleotide 605. Also located upstream of the coding region at positions 423 and 446 and at positions 330 and 355, reasonable −10 and −35 promoter elements, recognized by RNA polymerase containing the σ^{70} subunit can be assigned on the basis of their consensus sequences (ref. 24; see Fig. 5*B*). Preliminary evidence indicates that transcription start sites are located at positions 457, 458, and 508 by primer-extension experiments (data not shown).

Mapping of *cspA*. *cspA* was mapped to 79 minutes of the *E. coli* chromosome using the Kohara λ phage clone library (25). Nitrocellulose filters containing the phage library were prepared from lysates. Hybridization with either an oligonucleotide or nick-translated probe from sequences within *cspA* clearly yielded one dark spot on an autoradiogram (data not shown). The position of the spot on the autoradiogram corresponds to the location of the gene on the *E. coli* chromosome. The clone containing the *cspA* gene is 9F6 (602) according to Kohara *et al.* (25).

DISCUSSION

CS7.4 is unique among *E. coli* proteins that exhibit increased synthesis in the cold in that it is not detected at normal growth temperatures (30°C or 37°C; see ref. 8). Nevertheless, at its maximum rate, shortly after temperature shift, CS7.4 becomes the most abundantly synthesized protein in the cell. The stability of the protein and the fact that the protein continues to be synthesized after the initial burst of induction at 15°C indicate that a constant level of the protein is probably maintained in the cell as long as it remains exposed to cold temperatures. Although the protein does not appear to be actively degraded upon return to 37°C, the level of the protein is probably rapidly lowered by cell division in the absence of further synthesis.

Among several cold-induced proteins previously detected is one designated F10.6 (8). This protein is probably identical to the present cold shock protein, although there is a 3.1-kDa discrepancy in the reported molecular mass.

There are several possible functions of CS7.4. When *E. coli* cells growing at 37°C are shifted to 5°C, protein synthesis slowly decreases for 1 hr and then ceases (26). The accumulation of 70S ribosomes that were found after such a temperature shift has been interpreted to indicate a block in initiation of translation (26). A shift to 10°C, on the other hand, results in a growth lag of 4 hr followed by renewed growth (8, 27). It has, therefore, been suggested that since some of the proteins that are induced during cold shock are involved with protein synthesis, the response may prepare cells to reinitiate protein synthesis during exposure to cold temperatures (8). CS7.4 may be involved in such an adaptive process.

Probably the most compelling hypothesis at the current moment is that CS7.4 serves as an antifreeze protein. Antifreeze proteins are low molecular mass proteins commonly

found at high concentration in the serum of polar-dwelling marine fishes (28) and in the hemolymph of insects that winter in subfreezing climates (29). The fish antifreeze proteins may be divided into four classes based on structural properties (28). These include the glycoprotein, alanine-rich, and cysteine-rich classes, as well as a fourth class that is neither alanine- nor cysteine-rich. CS7.4 bears no similarity to any of these groups except with respect to its small size and its considerable proportion of hydrophilic residues. The non-glycoprotein fish antifreeze proteins, and to an even greater extent the insect thermal hysteresis (antifreeze) proteins, have a high proportion of hydrophilic residues that are thought to be important in hydrogen binding to the lattice of nascent ice crystals (29, 30). The crystallographic structure of the alanine-rich antifreeze polypeptide from the fish winter flounder has been determined (30). This 36-residue polypeptide appears to consist of a single α-helix extending throughout the entire molecule. The significance of this structure may lie in the ability of the helix dipole to align antiparallel with the hydrogen bond dipoles on the surface of the ice lattice (30). The possibility, as was suggested in the *Results* section, that CS7.4 may be largely α-helical is significant since it may indicate homology at the level of function and secondary structure that is not represented in the primary structure. Although there is as yet no direct evidence on the function of CS7.4, preliminary data indicate that either CS7.4 or another activity similarly induced at low temperature may protect *E. coli* from damage due to ice crystal formation during freezing. When cell cultures grown at 37°C were frozen and thawed after preincubation at 10°C for 6 hr, there was as much as a 70-fold increase in survival compared to cells frozen and thawed without the 10°C preincubation. Confirmation of the function of this protein obviously must await the mutational inactivation of the gene.

The rapid induction of CS7.4 synthesis suggests intriguing questions concerning the mechanism of regulation of *cspA*. Of the 13 polypeptides identified as being cold-induced (8), only CS7.4 is not detectable under normal growth conditions. Furthermore, the relative rate of synthesis of CS7.4 is far greater than that of other cold-induced proteins. The mode of regulation must also be very sensitive since a temperature shift as small as 5°C, from 30°C to 25°C, induces synthesis of the protein (see Fig. 1).

The transitory nature of *cspA* expression suggests a regulatory loop in which a product of the cold-shock regulon feeds back to inhibit further high-level synthesis of CS7.4. Autogenous regulation, in which the CS7.4 protein represses its own synthesis, would be a version of this general model. It should also be noted that there is a short inverted repeat present in the upstream region between nucleotides 205 and 221, which may also constitute a binding site for a regulatory protein (see Fig. 5*B*).

Recent primer extension experiments from this laboratory indicate that levels of *cspA* transcripts increase and then subsequently decrease upon shift to low temperature in a manner corresponding to the induction kinetics of the CS7.4 protein shown in Fig. 2 (data not shown). Obviously, much work needs to be done to elucidate the mode of regulation of the *cspA* gene as well as the function of the CS7.4 protein.

We are grateful to Dr. Sumiko Inouye for providing excellent technical advice and Dr. Jodi Fausnaugh for assistance with automated Edman degradation. Thanks also to Mei-yin Hsu for making the filters for the *E. coli* library and Dr. Ken Rudd for providing the Kohara library.

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